Erm(41)-dependent inducible resistance to azithromycin and clarithromycin in clinical isolates of Mycobacterium abscessus

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Abstract: OBJECTIVES: The ribosomal methylase Erm(41) confers inducible resistance to macrolides in Mycobacterium abscessus. The aim of this work was to systematically study and compare drug susceptibility to clarithromycin and azithromycin in M. abscessus and Mycobacterium chelonae clinical isolates with a particular focus on inducible drug resistance. METHODS: Clinical isolates of M. abscessus subsp. abscessus (n = 21), M. abscessus subsp. bolletii (n = 16), M. abscessus subsp. massiliense (n = 10) and M. chelonae (n = 22) were characterized regarding their erm(41) and rrl genotypes and subjected to drug susceptibility testing (DST) for clarithromycin and azithromycin. Microdilution DST was performed in cation-adjusted Mueller-Hinton broth (pH 7.4) with readings at days 3, 7 and 12 and with pre-incubation at subinhibitory macrolide concentrations for erm(41) induction. In addition, the influence of variations in pH and growth medium on DST results was examined. RESULTS: MICs of azithromycin were consistently higher than those of clarithromycin. In strains with an inducible erm(41) gene, high median MICs of 256 mg/L on day 12 were observed for both clarithromycin and azithromycin. Inducible resistance was at least as pronounced for azithromycin as for clarithromycin. CONCLUSIONS: Our findings do not support the suggestion of a preferential use of azithromycin over clarithromycin in order to limit inducible macrolide resistance. Both compounds provoked a comparable resistance phenotype in M. abscessus. Caution is needed when using either azithromycin or clarithromycin for treatment of M. abscessus infections.

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Erm(41)-dependent inducible resistance to azithromycin and clarithromycin in clinical isolates of *Mycobacterium abscessus*

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Objectives: The ribosomal methylase Erm(41) confers inducible resistance to macrolides in *Mycobacterium abscessus*. The aim of this work was to systematically study and compare drug susceptibility to clarithromycin and azithromycin in *M. abscessus* and *Mycobacterium chelonae* clinical isolates with a particular focus on inducible drug resistance.

Methods: Clinical isolates of *M. abscessus* subsp. *abscessus* ($n=21$), *M. abscessus* subsp. *bolletii* ($n=16$), *M. abscessus* subsp. *massiliense* ($n=10$) and *M. chelonae* ($n=22$) were characterized regarding their *erm*(41) and *rrl* genotypes and subjected to drug susceptibility testing (DST) for clarithromycin and azithromycin. Microdilution DST was performed in cation-adjusted Mueller–Hinton broth (pH 7.4) with readings at days 3, 7 and 12 and with pre-incubation at subinhibitory macrolide concentrations for *erm*(41) induction. In addition, the influence of variations in pH and growth medium on DST results was examined.

Results: MICs of azithromycin were consistently higher than those of clarithromycin. In strains with an inducible *erm*(41) gene, high median MICs of ≥256 mg/L on day 12 were observed for both clarithromycin and azithromycin. Inducible resistance was at least as pronounced for azithromycin as for clarithromycin.

Conclusions: Our findings do not support the suggestion of a preferential use of azithromycin over clarithromycin in order to limit inducible macrolide resistance. Both compounds provoked a comparable resistance phenotype in *M. abscessus*. Caution is needed when using either azithromycin or clarithromycin for treatment of *M. abscessus* infections.

Keywords: macrolides, rapidly growing mycobacteria, inducible resistance, drug susceptibility testing

Introduction

Azithromycin is an orally administered azalide antimicrobial that is structurally related to macrolides. The serum concentration of azithromycin is lower than that of clarithromycin and erythromycin,[1] as azithromycin is rapidly removed from the circulation and extensively distributed to intracellular compartments.[1–3] These unique pharmacokinetic properties of azithromycin allow for single daily dosages and intermittent treatment,[1] which can be beneficial for patient compliance, especially in the context of long-term regimens commonly required in mycobacterial infections.

Newer macrolides are considered key agents for the treatment of emerging infections caused by the closely related, rapidly growing mycobacterial species *Mycobacterium abscessus* and *Mycobacterium chelonae*.[4] In both species, acquired resistance to macrolides occurs through point mutations at positions 2058 or 2059 of the 23S rRNA (*rrl*) gene.[5,6] In contrast to *M. chelonae*, *M. abscessus* harbours an inducible ribosomal methylase, Erm(41), which represents an additional intrinsic resistance mechanism against macrolides. Inducible resistance can be demonstrated either by pre-incubation for 3 days prior to drug susceptibility testing (DST) using subinhibitory macrolide concentrations between 0.125× and 0.5× MIC or by prolonging incubation up to 12–14 days.[5,7,8]

The subspecies designations within *M. abscessus sensu lato*, i.e. *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*, are the subject of an ongoing taxonomic debate.[5,9–13] *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* both usually carry an inducible *erm*(41) gene.[9] Recently, Bastian *et al.*[9] reported that a single nucleotide exchange, T28C (Trp-10→Arg), in *erm*(41) leads to a loss of methylase activity in *M. abscessus* subsp. *abscessus*, resulting in a phenotype that is naturally susceptible to macrolides. *M. abscessus* subsp. *massiliense* differs from *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* by an *erm*(41) that is dysfunctional due to two characteristic deletions (bases 64–65 and

159–432).[5,13] Interestingly, two strains of *M. abscessus* subsp. *massiliense* with a functional *erm*(41) gene have been described recently.[14]

Data comparing the efficacy of clarithromycin and azithromycin in rapidly growing mycobacteria (RGM) are scarce. Using the agar gradient diffusion method (Etest), Bastian *et al.*[9] reported that MICs of azithromycin for *M. abscessus* complex tend to be higher than those of clarithromycin. This is in accordance with an early report of Brown *et al.*,[15] who observed higher MICs of azithromycin compared with clarithromycin for *M. abscessus* and *M. chelonae* using broth microdilution assays. However, the procedures used for species identification in the latter study at that time do not reflect current state-of-the-art taxonomy.[15] In contrast with these studies, Choi *et al.*[16] more recently reported that azithromycin is a weaker inducer of *erm*(41) gene expression than clarithromycin and should therefore be preferred in antibiotic therapy of *M. abscessus* infections. The aim of this work was to systematically evaluate the *in vitro* drug susceptibility of clarithromycin and azithromycin in *M. abscessus* and *M. chelonae* clinical isolates, with emphasis on inducible resistance.

Materials and methods

Strains

Clinical isolates of *M. abscessus* subsp. *abscessus* ($n=21$), *M. abscessus* subsp. *bolletii* ($n=16$), *M. abscessus* subsp. *massiliense* ($n=10$) and *M. chelonae* ($n=22$) were isolated from patient specimens between 2005 and 2013. Species identification was based on the sequences of the 16S rRNA, the *rpoB* and the *erm*(41) genes as described previously.[13,17,18] The *erm*(41) and *rrl* genotypes were determined by DNA sequence analysis as described previously.[19] In brief, the amplification primers used were MCLR 19F (5′-GTAGCGAAATTCCTTGTCGG-3′, *E. coli rrl* positions 1930–1949) and MCLR 21R (5′-TTCCCGCTTAGATGCTTTCAG-3′, *E. coli rrl* positions 2765–2745) for the *rrl* gene and erm41f2 (5′-GACCGGGGCCTTCTT CGTGAT-3′) and erm41r2 (5′-GACTTCCCCGCACCGATTCC-3′) for *erm*(41). The PCR conditions for the *rrl* PCR were 3 min at 95°C, followed by 30 cycles at 95°C for 60 s, 48°C for 60 s and 72°C for 120 s. The PCR conditions for *erm*(41) were 5 min at 95°C, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. Sequencing was performed using an Applied Biosystems 3130 Genetic Analyzer and BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems, Carlsbad, CA, USA). The sequences were analysed using Lasergene SeqMan software (DNASTAR, Madison, WI, USA), the SmartGene database (SmartGene Zug, Switzerland) and the BLAST algorithm (http://blast.ncbi.nlm.nih.gov). To identify mutations in *rrl*, sequences were compared with the published *M. abscessus* subsp. *abscessus* genome (GenBank accession number NC_010397.1). The published sequences of *M. abscessus* subsp. *abscessus* strains ATCC 19977 (T28 sequevar, GenBank accession number FJ358483.1) and CR5701 (C28 sequevar, GenBank accession number HQ127366.1) were used as references for *erm*(41) analysis.

DST

DST was performed based on CLSI document M24-A2.[20] Antimicrobial compounds (Sigma–Aldrich, Buchs, Switzerland) were dissolved in DMSO (clarithromycin) or ethanol (azithromycin), filter sterilized, aliquotted into stock solutions of 5 g/L and stored at −80°C. Working solutions (256 mg/L, maximum final solvent concentration 5% v/v) were prepared by diluting thawed aliquots of the respective stock solutions in cation-adjusted Mueller–Hinton broth (CAMHB; Becton Dickinson, Allschwil, Switzerland) at pH 7.4. Two-fold serial dilutions of the working solutions were prepared in CAMHB using sterile 96-well microtitre plates (Greiner Bio-One, St Gallen, Switzerland). Visible precipitates indicative of decreased solubility of azithromycin or clarithromycin were not observed. Drug-free solutions were tested to exclude inhibitory activity of the solvents alone. For all antibiotics, a growth control containing no antibiotic and a negative control containing only CAMHB were included. *Mycobacterium peregrinum* ATCC 700686 was used as a quality control (QC) strain to test each new lot of antibiotic stock solutions. QC ranges were applied as recommended by the CLSI.[20] In addition, *M. abscessus* ATCC 19977 was tested with each new lot of antimicrobials as an internal QC.

For inoculum preparation, colonies were taken from fresh pure cultures grown on Luria-Bertani (LB) agar using sterile cotton swabs and transferred into glass vials containing 2 mL of sterile saline. Bacterial suspensions were adjusted to a turbidity equivalent to that of a ≥0.5 McFarland standard and diluted in CAMHB to generate a final inoculum suspension of $1–5\times10^5$ cfu/mL. Each inoculum solution was checked for purity and correct concentration by obtaining cfu counts of suitable dilutions plated on LB agar. After inoculation, microdilution plates were covered with adhesive seals and incubated at 37°C for *M. abscessus sensu lato* or 30°C for *M. chelonae*. Growth was assessed by visual inspection of the microtitre plates after 3, 7 and 12 days of incubation. In accordance with Nash *et al.*,[5] a clarithromycin MIC of >32 mg/L at day 12 was used as the criterion for inducible macrolide resistance. For pre-incubation experiments, MIC testing was repeated following 3 days of pre-incubation of liquid cultures in 0.25× MIC of clarithromycin and azithromycin, respectively.[5,7,8] Prior to MIC testing, pre-incubated liquid cultures were pelleted and washed twice using CAMHB to remove residual antibiotics. For all MIC determinations using pre-incubated isolates, sequencing of the *erm*(41) gene was performed before and after the 3 day pre-incubation period as well as at day 12 of MIC testing in order to exclude the simultaneous presence of mixed C28 and T28 populations or a potential selection of the T28 genotype.

Results

DST

Using the broth microdilution method based on the current CLSI recommendations,[20] we determined MIC ranges and median MICs of clarithromycin and azithromycin for a set of clinical RGM isolates: *M. abscessus* subsp. *abscessus* (T28 sequevars), $n=21$; *M. abscessus* subsp. *bolletii*, $n=16$; *M. abscessus* subsp. *massiliense*, $n=10$; and *M. chelonae*, $n=22$ (Table 1). The data show that the MICs of azithromycin were consistently higher than those of clarithromycin for all examined isolates. In strains carrying an inducible Erm(41) methylase, high median MICs of both clarithromycin and azithromycin were observed at day 7 (azithromycin >256 mg/L and clarithromycin 32 mg/L for *M. abscessus* subsp. *abscessus* T28 sequevars; azithromycin >256 mg/L and clarithromycin 128 mg/L for *M. abscessus* subsp. *bolletii*).

Assessment of inducible macrolide resistance

We additionally studied the effect of pre-incubation with macrolides on selected *M. abscessus* isolates. For *M. abscessus* subsp. *abscessus* ATCC 19977 and a clinical *M. abscessus* subsp. *abscessus* isolate [both *erm*(41) T28 sequevars], pre-incubation at subinhibitory concentrations of either clarithromycin or azithromycin resulted in a marked increase in the MICs of both compounds between days 3 and 12 (Figure 1a and b). Inducible resistance was at least as pronounced against azithromycin as against clarithromycin in *M. abscessus* subsp. *abscessus erm*(41) T28 sequevars, regardless of pre-incubation (Figure 1a and b). A similar but much less pronounced increase in MIC values was observed for

Table 1. Clarithromycin and azithromycin DST results of *M. abscessus* complex and *M. chelonae* clinical isolates

	MIC range (mg/L)			Median MIC (mg/L)		
Species/antibiotic	day 3	day 7	day 12	day 3	day 7	day 12
M. abscessus subsp. *abscessus*						
clarithromycin ($n=21$)	<0.5–8	<0.5–256	32 to >256	0.5	32	256
azithromycin ($n=21$)	<0.5–256	64 to >256	256 to >256	4	>256	>256
M. abscessus subsp. *bolletii*						
clarithromycin ($n=16$)	1–4	32–256	256 to >256	1	128	256
azithromycin ($n=16$)	2–128	256 to >256	>256	32	>256	>256
M. abscessus subsp. *massiliense*						
clarithromycin ($n=10$)	<0.5	<0.5–2	<0.5–4	<0.5	<0.5	<0.5
azithromycin ($n=10$)	1–4	2–8	2–32	4	8	8
M. chelonae						
clarithromycin ($n=22$)	<0.5–1	<0.5–2	<0.5–4	<0.5	<0.5	1
azithromycin ($n=22$)	<0.5–16	1–16	2–32	2	8	16

Shown are MICs for isolates with a wild-type *rrl* gene. *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* isolates carried an inducible Erm(41) methylase (all T28 sequevars).

the two *M. abscessus* subsp. *abscessus erm*(41) C28 sequevars, again regardless of pre-incubation (Figure 1c and d). Azithromycin and clarithromycin MIC readings on day 12 were below or around 16 and 2 mg/L, respectively, and thus much lower than those for the two T28 sequevars (both >256 mg/L, Figure 1a and b). Interestingly, the day 12 azithromycin values for the two C28 sequevars were clearly higher than the day 12 azithromycin MICs for clinical *M. abscessus* subsp. *massiliense* isolates, which carry a large deletion in the *erm*(41) gene (see Figure 1e). A control clinical *M. abscessus* subsp. *abscessus* isolate carrying the *rrl* mutation A2058G showed clarithromycin and azithromycin MICs of >256 mg/L already on day 3, regardless of pre-incubation (Figure 1f).

Effect of the medium composition on the MICs of clarithromycin and azithromycin

The two liquid media used for DST in the previous studies differ both in their composition and acidity.[9,16] Bastian *et al.*[9] employed the widely used CAMHB (pH 7.4) medium, which contains beef extract, hydrolysed casein, starch and defined amounts of Ca^{2+} and Mg^{2+}. Choi *et al.*[16] used Middlebrook 7H9 medium, which is a complex liquid growth medium frequently used for the cultivation of mycobacteria. Middlebrook 7H9 has a lower pH (pH 6.8) and contains a large number of inorganic salts. Since macrolide activity is sensitive to pH,[1,20] we hypothesized that the use of the different media may influence the MIC values. Clarithromycin MICs for *M. abscessus* subsp. *abscessus* ATCC 19977 on days 3, 7 and 12 were 4, 4 and 32 mg/L in Middlebrook 7H9 and 1, 8 and 256 mg/L in CAMHB, respectively. For the same strain, the observed azithromycin MICs were 8, 256 and >256 mg/L in Middlebrook 7H9 and 2, 64 and 256 mg/L in CAMHB, respectively. Azithromycin consistently showed an inducible resistance phenotype comparable to that of clarithromycin also at additional pH levels (pH 6.1 and 8.1) in both media (data not shown).

Discussion

Macrolides such as clarithromycin and azithromycin are important components of therapeutic regimens against infections caused by *M. abscessus sensu lato* and *M. chelonae*. Proper identification of *M. abscessus* isolates to the subspecies level is indispensable, since significant differences exist in natural resistance to macrolides due to a functional, dysfunctional or non-existent *erm*(41) gene.[5,9] Recently, Bastian *et al.*[9] investigated a significant number of *M. abscessus* complex clinical isolates by phenotypic DST for clarithromycin and azithromycin and by sequencing of the *erm*(41) gene. These results indicated that MICs of azithromycin were generally higher than those of clarithromycin after 5 and 7 days of incubation.[9] In contrast, experiments conducted more recently by Choi *et al.*[16] in *M. abscessus* subsp. *abscessus* indicated that clarithromycin was a stronger inducer of *erm*(41), leading to higher mRNA expression and a more rapid increase in MICs during prolonged incubation compared with azithromycin.

In order to resolve these apparently conflicting results, we reanalysed the methodology used in the published studies and conducted additional experiments. We have identified significant methodological differences between the above-mentioned studies and the current CLSI guideline, which recommends broth microdilution in CAMHB for routine DST of RGM.[20] Bastian *et al.*[9] used a commercial broth microdilution method (with prolonged incubation for inducible macrolide resistance) for clarithromycin and the agar gradient diffusion method (Etest) for azithromycin to compare MIC ranges and mean MICs of the two drugs. Choi *et al.*[16] used pre-incubation and prolonged incubation in Middlebrook 7H9 medium (pH 6.8) to evaluate differences in inducible resistance between clarithromycin and azithromycin.

Our data using CAMHB (pH 7.4) and test conditions based on the CLSI guidelines show that the median MICs of azithromycin for *M. abscessus sensu lato* and *M. chelonae* are markedly higher

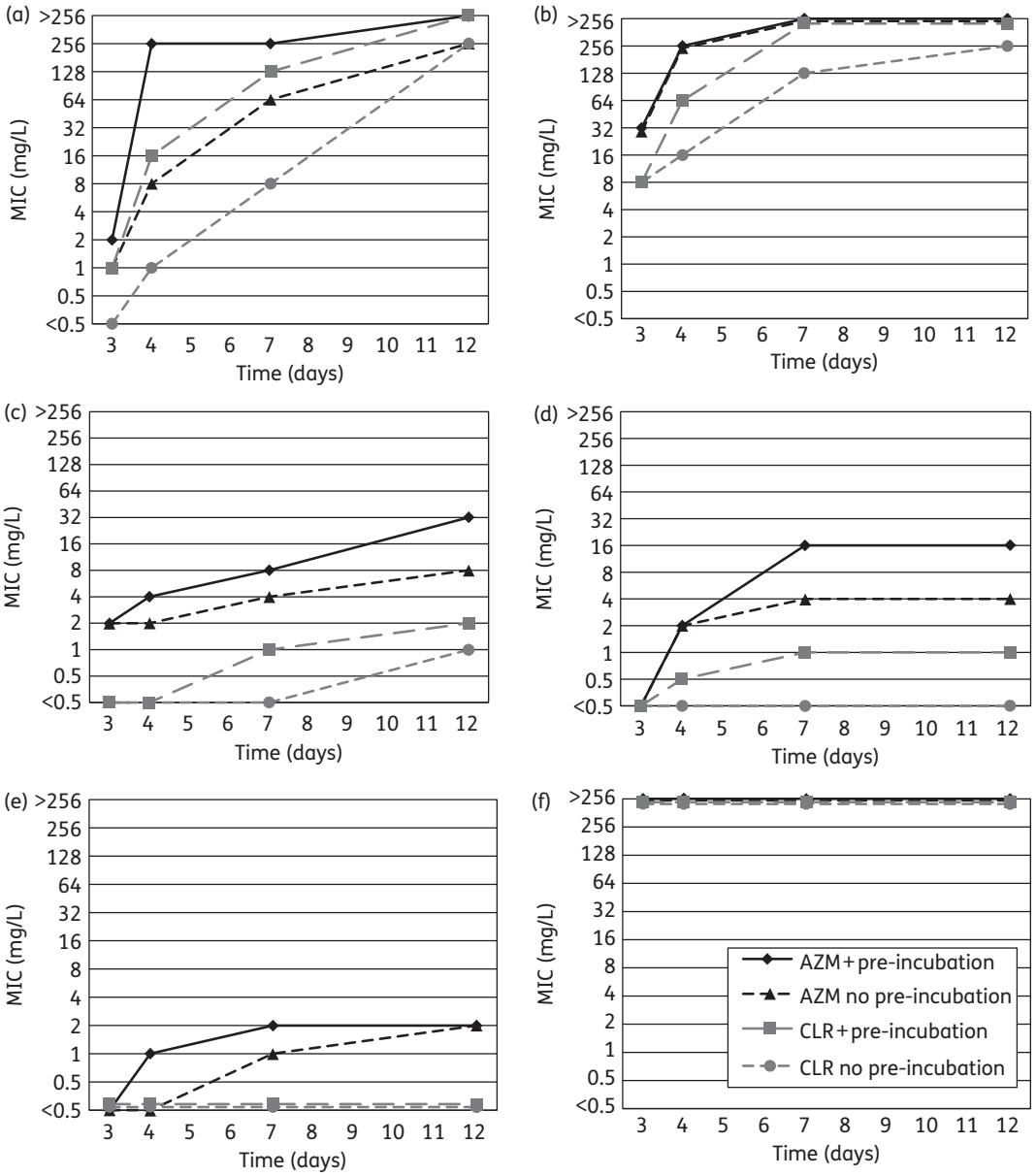

Figure 1. MICs of clarithromycin (CLR) and azithromycin (AZM) for *M. abscessus* complex isolates. (a) *M. abscessus* subsp. *abscessus* ATCC 19977, *erm*(41): T28, *rrl*: wild-type. (b) *M. abscessus* subsp. *abscessus* clinical isolate, *erm*(41): T28, *rrl*: wild-type. (c and d) *M. abscessus* subsp. *abscessus* clinical isolates, *erm*(41): C28, *rrl*: wild-type. (e) *M. abscessus* subsp. *massiliense*, *erm*(41): two deletions (2 and 276 bp), *rrl*: wild-type. (f) *M. abscessus* subsp. *abscessus* clinical isolate, *erm*(41): T28, *rrl*: A2058G. For pre-incubation experiments, MIC testing was repeated following 3 days of pre-incubation of liquid cultures in 0.25× MIC of clarithromycin and azithromycin, respectively.

than those of clarithromycin. Inducible resistance was comparable or even slightly more pronounced for azithromycin than for clarithromycin in both *M. abscessus* subsp. *abscessus erm*(41) T28 sequevars and *M. abscessus* subsp. *bolletii* (Table 1). We conducted additional experiments with pre-incubation followed by prolonged incubation using representative strains belonging to the *M. abscessus* complex and compared the results with those from our standard testing system (prolonged incubation alone). In contrast to the recent report of Choi *et al.*,[16] our results do not show that clarithromycin triggers a more rapid or more pronounced *erm*(41)-related macrolide resistance than azithromycin in *M. abscessus sensu lato*. We observed a similar inducible resistance phenotype for both compounds, despite the generally higher MICs of azithromycin compared with clarithromycin (Table 1 and Figure 1). We hypothesized that differences in growth media, e.g. pH, might influence azithromycin activity, which might explain the different results by Choi *et al.*[16] However, additional experiments in Middlebrook 7H9 and standard CAMHB medium ruled out this possibility.

We also observed that *M. abscessus* subsp. *abscessus erm*(41) C28 sequevars showed increasing MIC levels during prolonged incubation with clarithromycin. This observation is supported by

the previous finding that both T28 and C28 sequevars show a comparable increase in mRNA transcripts upon exposure to macrolides.[5] The MICs of clarithromycin and azithromycin did not increase to >32 mg/L at day 12 and thus were much lower than those for *M. abscessus* subsp. *abscessus* T28 sequevars. However, these MICs were clearly higher than the macrolide MICs for *M. abscessus* subsp. *massiliense*, which possesses an *erm*(41) with a large deletion (Table 1). These results suggest that the single amino acid exchange, Trp-10→Arg (T28C), does not completely abort Erm(41) functionality and that a deletion as found in *M. abscessus* subsp. *massiliense* is required to result in a completely dysfunctional gene product. However, since only two *M. abscessus* subsp. *abscessus* C28 sequevars were available to us, this observation will require further clarification on a larger set of such strains.

In conclusion, the median MICs of azithromycin were higher and increased at a similar rate during prolonged incubation compared with those of clarithromycin for clinical *M. abscessus* complex isolates. Variation of our standard test conditions with respect to pre-incubation, pH and medium did not support the notion that azithromycin is a weaker inducer of *erm*(41)-related macrolide resistance in *M. abscessus sensu lato* than clarithromycin.[16] Our data show that the single amino acid exchange Trp-10→Arg in *erm*(41) does not result in a complete loss of inducibility, although MICs of both macrolides after prolonged incubation remained well below those for *erm*(41) T28 sequevars. Our data further show that both clarithromycin and azithromycin may trigger inducible macrolide resistance *in vitro* and that caution is advisable when using either compound *in vivo* in the presence of a functional Erm(41) methylase.

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